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Use of a series of chemostat cultures to isolate 'improved' variants of the Quorn® myco-protein fungus, *Fusarium graminearum* A3/5

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Variants (designated A23-S and A24-S) of the Quorn® myco-protein fungus, *Fusarium graminearum* A3/5 were isolated from a series of glucose-limited cultures grown at a dilution rate of 0.18 h⁻¹ for a combined total of 109 d. These variants had unchanged mycelial morphologies but, when grown in mixed culture with the parental strain (A3/5) in glucose-limited chemostat culture at 0.18 h⁻¹, A23-S and A24-S had selection coefficients of 0.013 and 0.017 h⁻¹, respectively, and supplanted A3/5. When a monoculture of A23-S was grown in a glucose-limited culture at a dilution rate of 0.18 h⁻¹, the appearance of highly branched (so-called colonial) mutants was delayed compared with their appearance in chemostat cultures of the parental strain. Furthermore, when a monoculture of A24-S was grown in glucose-limited culture at 0.18 h⁻¹, the appearance of colonial mutants was delayed even further. Thus, it is possible to isolate advantageous (relative to A3/5) variants of *F. graminearum* A3/5 which have unchanged mycelial morphologies, but in which the appearance of colonial mutants is delayed.

Keywords: *Fusarium graminearum* A3/5, myco-protein, chemostat culture, colonial mutants, periodic selection

INTRODUCTION

Novick & Szilard (1950), Moser (1958) and Powell (1958) recognized that an organism cultured under constant conditions in a chemostat adapted to its environment by mutation and natural selection. Subsequently, chemostats have been used by accident or design to isolate 'improved' strains of bacteria (Tsang & Grootwassink, 1988; Silman *et al.*, 1989; Cheng *et al.*, 1989; Mikkola & Kurland, 1992; Laci & Lawford, 1992), yeasts (Downie & Garland, 1972; Francis & Hansche, 1973; Brown & Oliver, 1982) and filamentous fungi (Forss *et al.*, 1974; Righelato, 1976; Solomons, 1985; Wiebe *et al.*, 1991). In the case of filamentous fungi, selectively advantageous mutants (so-called colonial mutants) were recognized because they were more highly branched than their parental strains. To date, no mutants of filamentous fungi with improved growth characteristics but unaltered mycelial morphologies have been isolated although their existence can be predicted from the periodic selection (Dykhuizen & Hartl, 1983) observed in chemostat populations of *Fusarium graminearum* A3/5; changes in the proportion of cycloheximide-resistant to cycloheximide-sensitive propagules in chemostat populations indicated that ad-

vantageous, cycloheximide-sensitive variants of unaltered morphology replaced the parental population even before colonial mutants appeared (Wiebe *et al.*, 1993). The isolation of advantageous variants with unaltered mycelial morphologies is of considerable commercial interest because, in the production of Quorn® myco-protein, fermentations of *F. graminearum* have to be terminated as soon as highly branched mutants appear (these mutants cause alterations to both the filtration characteristics and the texture of the final product – it becomes more 'crumbly'), with a consequent decrease in productivity (Trinci, 1992).

In this paper, we describe how a series of chemostats was used to isolate two 'improved' variants of *F. graminearum* A3/5 (i.e. variants which have a selective advantage over A3/5) which have an unaltered mycelial morphology. The selective advantage of a third variant which is more sparsely branched than A3/5, is also described.

METHODS

Organism and medium. *Fusarium graminearum* Schwabe strain A3/5 was obtained from Mr T. W. Naylor, Marlow Foods, Billingham, UK. Stock cultures were maintained as macro-

conidia at -70°C in 20% (v/v) glycerol. Macroconidia for inocula were prepared as described by Wiebe *et al.* (1991).

The defined medium of Vogel (1956) was used, with glucose as the carbon source instead of sucrose. For submerged cultures, $1.65\text{ g }(\text{NH}_4)_2\text{SO}_4\text{ l}^{-1}$ was substituted for $2\text{ g NH}_4\text{NO}_3\text{ l}^{-1}$ as the nitrogen source. Vogel's mineral salts solution was prepared at $50\times$ final concentration, sterilized by membrane ($0.2\text{ }\mu\text{m}$ diam. pore size) filtration and added to the sterile glucose solution. Glucose solutions [final concentration of $3\text{ g glucose (l medium)}^{-1}$] for chemostat cultures were prepared in 10 l volumes and autoclaved for 60 min at 121°C ; no caramelization and no significant loss of glucose was observed. For plate cultures, Vogel's modified medium containing $10\text{ g glucose l}^{-1}$ and $3\text{ g }(\text{NH}_4)_2\text{SO}_4\text{ l}^{-1}$ or $2\text{ g NH}_4\text{NO}_3\text{ l}^{-1}$ or $2\text{ g NaNO}_3\text{ l}^{-1}$ was solidified with agar (Davis Gelatine; 15 g l^{-1} , final concentration). For media to detect resistant strains, cycloheximide ($250\text{ }\mu\text{M}$) or potassium chlorate (300 mM) was added to modified Vogel's medium. All cultures were incubated at 25°C .

Chemostat cultures. Cultures were grown at a dilution rate of 0.18 h^{-1} at 25°C (under the culture conditions employed, A3/5 has a D_{crit} of about 0.23 h^{-1}) in a 2 l Braun Biosat M fermenter (B. Braun Medical Ltd.) as described by Wiebe & Trinci (1991). Biomass retention in the fermenter vessel was monitored daily by taking dry weight measurements of samples both from inside the fermenter vessel and from the overflow. No retention of biomass in the vessel was observed.

Inoculation protocols and isolation of variants. Variant A21-XS was isolated from a series of glucose-limited chemostat cultures grown at a dilution rate of 0.18 h^{-1} (M. G. Wiebe, G. D. Robson, S. G. Oliver and A. P. J. Trinci, unpublished results). Chemostat AC20 was inoculated with macroconidia harvested from spread plates of *F. graminearum* A3/5 following the procedure of Wiebe & Trinci (1991): a steady state was first achieved in such chemostats 24–48 h after onset of continuous flow. A sample was taken from chemostat AC20 after 720 h (196 generations) of cultivation and macroconidia (separated from the mycelial biomass by filtration through two layers of sterile lens tissue; Whatman No. 105) from this sample were diluted and used to prepare spread plates of agar-solidified modified Vogel's medium. After about 10 d incubation, plates which contained no colonial mutant colonies were retained and macroconidia were harvested from these plates and used to inoculate fresh spread plates (to ensure that no colonial mutants were present in the final inoculum). This second set of plates was used to produce macroconidia to inoculate the second chemostat (AC22) in the series. At 312 h (89 generations) after the onset of continuous flow, 8 ml of a suspension containing mycelia and conidia from chemostat AC22 was used to inoculate a third glucose-limited chemostat (AC23). Variant A23-S was isolated from chemostat AC23 at 255 h after the onset of continuous flow; a highly branched, colonial mutant (A22-1) was present in the AC23 population at a very low concentration at this time. Variant A24-S was isolated from chemostat AC24 which had been inoculated with macroconidia of A23-S. Chemostat AC24 was maintained for 1273 h (total time since inoculation of the first chemostat in the series was 2608 h or 677 generations). Variants A23-S and A24-S were isolated by harvesting macroconidia from the margins of colonies (about 5 d old) which had been inoculated centrally with a small drop (about $50\text{ }\mu\text{l}$) of a macroconidial and mycelial suspension from the fermenter population. By harvesting macroconidia from the margins of the colony, any colonial macroconidia present in the central part of the colony could be avoided (only mycelia of sparsely branched variants would be present at the colony margin). This method of isolating sparsely branched mutants was more rapid and efficient than the method (diluting macroconidia and

screening spread plates) described above for producing inoculum for chemostat AC22. Single-sporing macroconidia of each variant isolated confirmed that colonial mutants were absent. The procedure used to isolate the variants A23-S, A24-S and A22-1 is shown in Fig. 1.

Marking mutants for competition experiments. Chlorate-resistant mutants of strains A3/5, A23-S, A24-S and A21-XS were selected by inoculating plates of modified Vogel's medium containing 300 mM potassium chlorate with about 5×10^4 macroconidia and subsequently isolating chlorate-resistant colonies. Each isolate was subcultured onto plates of Vogel's medium containing NaNO_3 as the sole nitrogen source, as well as onto Vogel's medium containing NH_4NO_3 . Only nitrate-non-utilizing strains were retained for the experiments described below. Chlorate-resistant mutants of *F. graminearum* A3/5 are very stable, with a reversion frequency of less than 1 in 10^7 macroconidia (G. D. Robson, unpublished results).

Monitoring of cycloheximide-resistant mutants and morphological (colonial) mutants in chemostat populations. Samples (about 10 ml) were removed daily from the fermenter vessel and macroconidia were separated from the mycelial biomass by filtration through two layers of sterile lens tissue. Counts of viable macroconidia were made on modified Vogel's medium (10 replicates per sample), as described by Wiebe *et al.* (1991). To test for spontaneous mutations giving rise to cycloheximide resistance, the macroconidia (about 3×10^4 per plate) were also inoculated onto medium containing $250\text{ }\mu\text{M}$ cycloheximide (10 replicates per sample). The plates were incubated for 3 d for viable counts, or 6–8 d to detect cycloheximide resistance.

Highly branched, colonial mutants were identified from their colonial morphologies (Trinci, 1992) and the proportion of colonial mutant(s) to parental strain was determined in the total population (i.e. macroconidia and mycelial fragments) as described by Wiebe *et al.* (1991).

Measurement of selection coefficients. In a chemostat, the selective advantage of one strain (e.g. a colonial mutant) compared with another, can be quantified by determining the selection coefficient, s (Dykhuizen & Hartl, 1981):

$$s = \frac{\ln\left(\frac{p_m}{q_m}\right) - \ln\left(\frac{p_0}{q_0}\right)}{t} \quad (1)$$

where p_m is the concentration of mutant strain at time t , q_m is the concentration of the parental strain at time t , and p_0 and q_0 are the initial concentrations of each strain. Selection coefficients were calculated for glucose-limited chemostat cultures inoculated with macroconidia of two different strains of *F. graminearum* and grown at a dilution rate of 0.18 h^{-1} . In such competitions, morphologically similar strains were distinguished by competing chlorate-resistant (nitrate-non-utilizing) and chlorate-sensitive (nitrate-utilizing) strains. Each competition was repeated with the chlorate resistance marking the other strain. Counts of colony forming units (derived from either mycelial fragments or macroconidia) were made on modified Vogel's medium containing NH_4NO_3 or NaNO_3 as the nitrogen source, and with NH_4NO_3 plus 300 mM chlorate (10 replicates per sample for each medium). The plates were incubated for 3 d and the proportion of chlorate-resistant and chlorate-sensitive propagules in the total population was determined. The proportion of nitrate-utilizing and nitrate-non-utilizing propagules was determined independently. With ammonium sulphate as the sole nitrogen source in the medium, chlorate resistance confers neither a selective advantage nor a

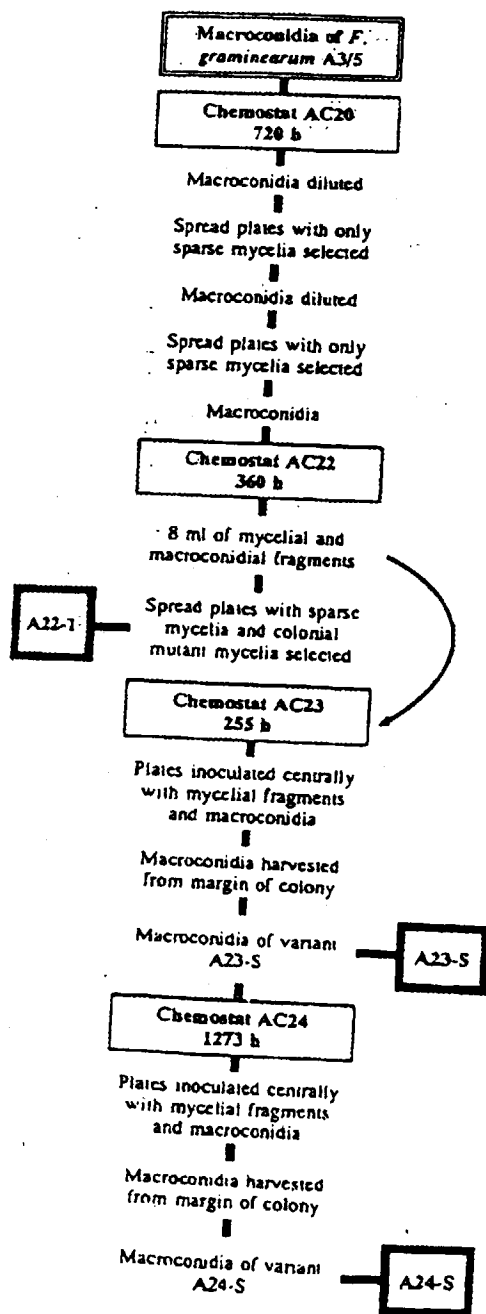


Fig. 1. Flow diagram showing the history of the chemostat cultures from which the A22-1 (isolated after 1080 h of evolution), A23-S (isolated after 1335 h of evolution) and A24-S

disadvantage in the labelled strain (M. G. Wiebe, unpublished result). The selection coefficient for each strain was measured both with and without the chlorate resistance marker to confirm the selective neutrality of chlorate resistance in the culture conditions used. It was not necessary to use chlorate-resistant strains to calculate selection coefficients for the colonial mutant A22-1 as highly branched mutants are easily distinguishable from sparsely branched strains (Wiebe *et al.*, 1992).

Measurements of mycelial morphology. Hyphal growth unit length (G) is a measure of mycelial branching (Trinci, 1974); these measurements were made on mid-exponential phase mycelia which had been grown for about 24 h in 50 ml volumes of medium in 250 ml Erlenmeyer flasks incubated on a rotary shaker (with a throw of 2.5 cm) at 200 r.p.m. Hyphal growth unit lengths were measured using a MeasureMouse system (Analytical Measuring Systems) and a Nikon Microscope linked to a video camera and an Amstrad 1512PC as described by Wiebe & Trinci (1991).

RESULTS

Morphology of variants A21-XS, A23-S and A24-S

Fig. 1 shows the protocol used to isolate variants A22-1, A23-S and A24-S. Variant A21-XS (isolated previously) was significantly more sparsely branched (longer G value) than the parental strain whilst the mycelial morphologies of variants A23-S and A24-S were indistinguishable (same G values) from that of the parental strain (Table 1).

Competition between the parental strain A3/5 and variant A21-XS

When grown in mixed culture in a glucose-limited chemostat culture at a dilution rate of 0.18 h^{-1} , variant A21-XS had a selection coefficient of $0.006 \pm 0.001 \text{ h}^{-1}$ relative to the parental strain (Fig. 2a).

Table 1. Hyphal growth unit lengths of *F. graminearum* A3/5, A21-XS, A23-S and A24-S in submerged culture

F. graminearum A3/5, A21-XS, A23-S and A24-S were grown at 25°C on modified Vogel's medium with $(\text{NH}_4)_2\text{SO}_4$ as the nitrogen source. The results are the means (\pm s.e.) of 25 replicates. Figures with the same superscript letter are not significantly different ($P < 0.05$).

Strain	Hyphal growth unit length in liquid medium (G , μm)
Parental strain (A3/5)	224 ± 10^a
A21-XS	274 ± 10^a
A23-S	190 ± 5^a
A24-S	201 ± 10^a

(isolated after 2608 h of evolution) variants were isolated. The figures give time in h after onset of continuous flow when samples were taken for plating. Variants A22-1, A23-S and A24-S supplanted A3/5 in glucose-limited chemostat cultures at a dilution rate of 0.18 h^{-1} with selection coefficients of 0.019 h^{-1} , 0.013 h^{-1} and 0.017 h^{-1} , respectively.

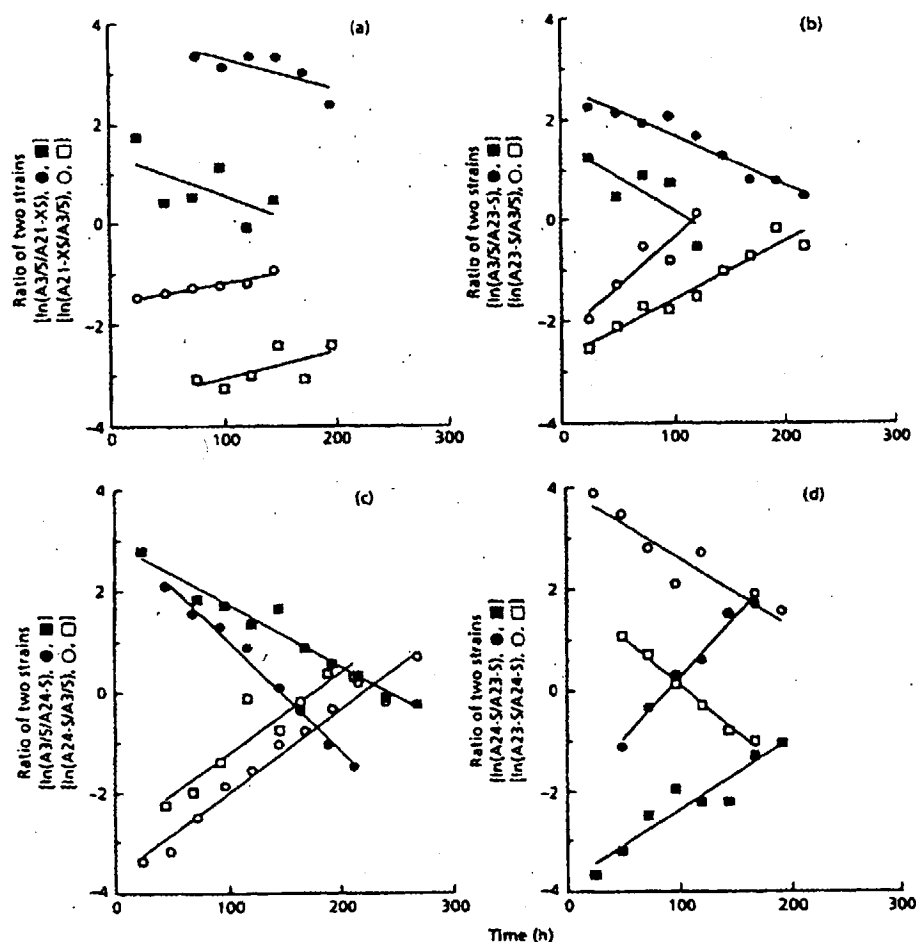


Fig. 2. Competition between chlorate-resistant (■, □) and chlorate-sensitive (●, ○) strains of *F. graminearum* grown in glucose-limited chemostat culture at a dilution rate of 0.18 h⁻¹. (a) competition between A3/5 (closed symbols) and A21-XS (open symbols), (b) competition between A3/5 (closed symbols) and A23-S (open symbols), (c) competition between A3/5 (closed symbols) and A24-S (open symbols) and (d) competition between A23-S (open symbols) and A24-S (closed symbols).

Competition between A3/5, A23-S and A24-S

The parental strain (A3/5) was grown in mixed culture with variants A23-S and A24-S. When mixed cultures were grown in a glucose-limited chemostat culture at a dilution rate of 0.18 h⁻¹, A23-S replaced A3/5 (Fig. 2b) with a selection coefficient of 0.013 ± 0.001 h⁻¹. Similarly, when mixed cultures were grown in a glucose-limited chemostat at a dilution rate of 0.18 h⁻¹, A24-S replaced A3/5 (Fig. 2c) with a selection coefficient of 0.017 ± 0.002 h⁻¹. The selective advantage of A24-S relative to A3/5 was significantly greater ($P < 0.05$, *t*-test) than the

selective advantage of A23-S relative to A3/5. When A23-S and A24-S were grown in glucose-limited chemostat culture at a dilution rate of 0.18 h⁻¹, A24-S replaced A23-S with a selection coefficient of 0.014 h⁻¹ (Fig. 2d).

Appearance of colonial mutants and periodic selection in an A23-S chemostat

A monoculture of variant A23-S was grown in glucose-limited chemostat culture at a dilution rate of 0.18 h⁻¹. Highly branched (colonial) mutants appeared in the population about 480 h or 124 generations after the onset

Table 2. Periods between peaks in the proportion of cycloheximide-resistant macroconidia in a population of *F. graminearum* A23-S

F. graminearum A23-S was grown in glucose-limited [$3 \mu\text{g glucose (l medium)}^{-1}$] chemostat culture on modified Vogel's medium (25°C , $\text{pH } 5.8 \pm 0.1$) at a dilution rate of 0.18 h^{-1} . Samples were removed at regular intervals and the proportion of cycloheximide (0.25 mM)-resistant macroconidia in the total macroconidial population was determined. This proportion was also determined for two subpopulations (sparsely branched and highly branched phenotypes).

Population	Time between peak maxima (h)						
	Peak 0-1	Peak 1-2	Peak 2-3	Peak 3-4	Peak 4-5	Peak 5-6	Peak 6-7
Total population	384	191	145	144	168	120	192
Sparsely branched mutant sub-population	384	191	145	144	168	96	216
Highly branched mutant sub-population					216	96	192

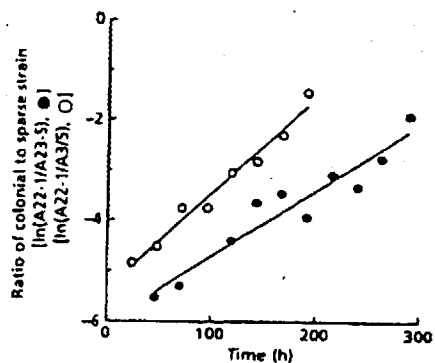


Fig. 3. Competition between *F. graminearum* strains A3/5 and colonial mutant A22-1 (○) and between A23-S and colonial mutant A22-1 (●) grown in glucose-limited chemostat culture at a dilution rate of 0.18 h^{-1} .

of continuous flow and replaced A23-S with a selection coefficient of 0.018 h^{-1} . Although the majority of the colonial population consisted of very highly branched mutants, less highly branched mutants were occasionally observed but never formed more than $2.4 \pm 0.9\%$ of the population. The selection coefficient (0.018 h^{-1}) for the colonial mutants that arose in the A23-S culture was comparable to the selection coefficients (0.017 – 0.034 h^{-1}) of colonial mutants which appeared in populations of the parental strain, A3/5 (Trinci, 1992). At about 937 h or 245 generations after the onset of continuous flow, highly branched (colonial) mutants formed $84 \pm 1.6\%$ of the population and then fluctuated between 80 and 90% of the total population until the experiment was terminated at 1273 h or about 334 generations after the onset of continuous flow.

Fluctuations occurred in the concentration of cyclo-

heximide-resistant macroconidia in the population and seven peaks of cycloheximide resistance were observed (Table 2). Excluding the first peak which occurred after 384 h or 100 generations, a mean interval of $160 \pm 12 \text{ h}$ (42 ± 3 generations) was observed between peaks in the cycloheximide-resistant population of macroconidia. Cycloheximide-resistant colonial mutants were first observed about 601 h or 156 generations after the onset of continuous flow, but they did not become dominant in the cycloheximide-resistant population until about 816 h or 213 generations (peak 4) after the onset of continuous flow. Fluctuations in cycloheximide resistance continued to be observed in populations of both A23-S and colonial mutant macroconidia (Table 2), indicating the appearance of advantageous mutants.

Appearance of colonial mutants and periodic selection in A24-S chemostats

A monoculture of variant A24-S was grown in a glucose-limited chemostat culture at a dilution rate of 0.18 h^{-1} . In the first experiment, highly branched (colonial) mutants appeared in the population about 600 h or 156 generations after the onset of continuous medium flow, and displaced the sparsely branched population with a selection coefficient of 0.023 h^{-1} . After 912 h or 237 generations of cultivation, the colonial population contributed $85.2 \pm 3.2\%$ of the total population and subsequently fluctuated between this and $96.4 \pm 0.8\%$ of the population until the fermentation was terminated at 1703 h or 442 generations after the onset of continuous flow. In a second experiment, no colonial mutants were observed in the A24-S population before the fermentation was terminated at 552 h or 135 generations after the onset of continuous flow.

Fluctuations in the concentration of cycloheximide-resistant macroconidia were monitored during the first A24-S experiment and seven peaks of cycloheximide were observed (data not shown). After the first interval, which lasted about 336 h or 87 generations, the average interval between peaks in the concentration of cycloheximide-

resistant macroconidia was 208 ± 28 h or about 54 generations, and was significantly ($P < 0.05$, *t*-test) longer than the intervals observed for either the A3/S (Wiebe *et al.*, 1993) or the A23-S chemostat populations (124 ± 12 h and 160 ± 12 h, respectively).

Competition between A3/S, A22-1 and A23-S

The parental strain (A3/S) and variant A23-S were each grown in competition with colonial mutant A22-1 in glucose-limited chemostat cultures at a dilution rate of 0.18 h^{-1} . Fig. 3 shows that the highly branched (colonial) mutant (A22-1) supplanted both sparsely branched strains but the parental strain (A3/S) was displaced more rapidly ($r = -0.019 \text{ h}^{-1}$) than A23-S ($r = -0.013 \text{ h}^{-1}$).

DISCUSSION

We have now isolated advantageous variants of *F. graminearum* with three different mycelial morphologies: variants more highly branched than A3/S (the colonial mutants); variants with a similar branching pattern to A3/S (A23-S and A24-S); and variants more sparsely branched than A3/S (A21-S).

When *F. graminearum* A3/S was grown in a glucose-limited chemostat culture at a dilution rate of 0.18 or 0.19 h^{-1} in three unperturbed cultures (i.e. cultures in which the steady-state was maintained throughout), colonial mutants first appeared 360, 386 and 421 h (99, 106 and 115 generations, respectively) after the onset of continuous flow; mean \pm SE of 389 ± 18 h or 107 ± 5 generations (Trinci, 1992; Wiebe *et al.*, 1991). However, in two perturbed chemostat cultures (in which steady states were not maintained throughout because in one the stirrer drive belt snapped and in the other the air supply became blocked and the dilution rate varied from 0.11 to 0.22 h^{-1}), colonial mutants first appeared at 481 h or 125 generations and 648 h or 177 generations after the onset of continuous flow. Since we have previously shown that perturbations in growth conditions (Wiebe *et al.*, 1992) and reductions in dilution rate (M. G. Wiebe, G. D. Robson, S. G. Oliver and A. P. J. Trinci, unpublished result) inhibit or delay the increase of colonial mutants in a population, comparisons between the appearance of colonial mutants in chemostat cultures of A3/S, A23-S and A24-S are made only for unperturbed A3/S cultures.

For glucose-limited chemostat cultures grown at a high dilution rate, appearance of colonial mutants in A23-S (at 480 h or 124 generations) and A24-S (at > 552 h or > 135 generations and 600 h or 160 generations) was delayed significantly compared with the parental strain (389 h or 107 generations). That variants of *F. graminearum* A3/S can be isolated in which the appearance of colonial mutants is delayed is of considerable commercial interest because, in the production of Quorn[®] myco-protein, industrial fermentations of *F. graminearum* have to be terminated as soon as colonial mutants appear (Trinci, 1992).

In glucose-limited chemostat culture, the interval between the onset of continuous flow and the first (adaptive) peak

of cycloheximide-resistant macroconidia was longer (384 h) for A23-S than that observed in the parental strain (longest interval = 171 h, Wiebe *et al.*, 1993), suggesting that the former culture was more homogenous at the onset of continuous medium flow than the latter. Similarly, a long first interval before the first (adaptive) peak of cycloheximide-resistant macroconidia was observed in both A24-S fermentations. Excluding this first interval, a mean interval of 160 ± 12 h (42 ± 3 generations) was observed between peaks in cycloheximide-resistant macroconidia for the A23-S culture. Furthermore, it may be significant that the mean interval between peaks in cycloheximide-resistant macroconidia for a glucose-limited chemostat culture of A24-S (208 ± 28 h) was longer ($P < 0.05$) than the mean interval (160 ± 12 h) observed for the A23-S culture. These periods are significantly longer ($P < 0.05$) than the mean interval between peaks in cycloheximide-resistant macroconidia in A3/S cultures (124 ± 12 h; Wiebe *et al.*, 1993). These results suggest that, although changes in the population occurred, in the A23-S and A24-S cultures, they occurred less frequently than in A3/S populations. Thus, the present results show that it is possible to isolate 'improved' variants (variants which have a competitive advantage when grown in mixed culture with A3/S and in which the appearance of colonial mutants is delayed compared with A3/S) of the Quorn[®] myco-protein fungus.

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